Conversion of big endothelin-1 to endothelin-1 by two types of metalloproteinases derived from porcine aortic endothelial cells

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Incubation of big endothelin-1 (big ET-1_{1-39}) with either the cytosolic or membrane fraction obtained from cultured endothelial cells, resulted in an increase in immunoreactive-endothelin (IR-ET), which was markedly inhibited by metal chelators. Phosphoramidon, a metalloproteinase inhibitor, specifically suppressed the membrane fraction-induced increase in IR-ET, whereas the increase in IR-ET observed with the cytosolic fraction was not influenced by phosphoramidon. Reverse-phase (RP)-HPLC of the incubation mixture of big ET-1 with the cytosolic or membrane fraction revealed one major IR-ET component corresponding to the elution position of synthetic ET-1_{1-21}. Simultaneously, immunoreactivities like the C-terminal fragment (CTF_{22-39}) of big ET-1 were present, as deduced from the RP-HPLC coupled with the radioimmunoassay for CTF. Our results indicate the presence of two types of metalloproteinases, which convert big ET-1 to ET-1 via a single cleavage between Trp^{21} and Val^{22}, in vascular endothelial cells.

Endothelin-1; Big endothelin-1; Metalloproteinase; Endothelium, vascular

1. INTRODUCTION

Endothelin-1 (ET-1) is a 21 amino acid peptide isolated from the culture medium of vascular endothelial cells (ECs) [1]. From the structure of prepro-form deduced from the nucleotide sequence of cDNA encoding ET-1, Yanagisawa et al. [1] proposed a biosynthetic pathway of ET-1, i.e. the prepro-form is initially processed by dibasic pair specific proteolysis to produce a 39 amino acid intermediate form, termed big ET-1, and the big ET-1 is converted to the mature form by an unusual proteolytic processing between Trp^{21} and Val^{22}. Since the vasoconstrictor activity of big ET-1 is much lower than that of ET-1 [2,3], the conversion from big ET-1 to ET-1 appears to be essential for the pathophysiological significance of ET-1. There is accumulating evidence that the C-terminal fragment (CTF_{22-39}) of big ET-1, as well as ET-1 and big ET-1, is present in the culture medium of ECs [4–6], hence ET-1 may be generated from big ET-1, via a single cleavage between Trp^{21} and Val^{22}, by a putative big ET-1 converting enzyme in ECs.

Most recently, we found that ET-1 was generated via a single cleavage between Trp^{21} and Val^{22} when incubating big ET-1 with the extract from cultured ECs at an acid pH [6]. Since the generation of ET-1 was specifically inhibited by pepstatin-A, we suggested that an aspartic proteinase was involved in the conversion of big ET-1 to ET-1 in vascular ECs [7]. We have also noted that a relatively small increase in ET immunoreactivity occurs during incubation of big ET-1 with the cell extract at pH 7.0 [7]. However, it remained to be determined whether this increase at the neutral pH was due to the conversion of big ET-1 to ET-1.

In the present study, we prepared both cytosolic and the membrane fractions from cultured ECs and investigated whether these fractions possess converting activity at neutral pH.

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2. MATERIALS AND METHODS

2.1. *Cell culture and preparation of cytosolic and membrane fractions*

ECs isolated from fresh porcine thoracic aortas were cultured, as described [8]. To obtain cytosolic and membrane fractions of ECs, the confluent cells after 5–9 passages were scraped with a Cell Lifter (Costar, MA). After washing with phosphate buffered saline, the cells were homogenized in ice-cold 20 mM Tris-HCl buffer (pH 8.0) containing 30 mM KCl, then the preparation was centrifuged at 105000 × *g* for 30 min. The resulting supernatant was used as the cytosolic fraction. The pellet was washed with the Tris-HCl buffer and resuspended in the same buffer, then the preparation was used as the membrane fraction.

2.2. *Incubation of big ET-1 with either cytosolic or membrane fractions*

Fifty μl of each fraction (derived from 4×10^5 cells) and 0.05 ml of enzyme inhibitor cocktail were mixed with 0.35 ml of 50 mM sodium phosphate buffer (pH 6.0–8.0). After preincubation at 37°C for 30 min, 0.05 ml of porcine big ET-1 solution (final concentration: 100 ng big ET-1/ml) was added to the mixture and the preparation was incubated at 37°C for 0.5–24 h. The reaction was stopped by boiling for 10 min. The samples were neutralized (to pH 7.4) with diluted NaOH or HCl and centrifuged at 8000 × *g* for 5 min. The resulting

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supernatant was diluted and served as sample for the radioimmunoassay (RIA) and reverse-phase (RP)-HPLC. Enzyme inhibitors used were bestatin (aminopeptidase inhibitor), E-64 (cysteine proteinase inhibitor), (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (*p*-APMSF, serine proteinase inhibitor), chymostatin (chymotrypsin inhibitor), phosphoramidon (metalloproteinase inhibitor) and thiorphan (neutral endopeptidase 24.11 inhibitor). Metal chelators, such as EDTA and 1,10-phenanthroline were also used. Thiorphan, EDTA and 1,10-phenanthroline were purchased from Sigma Chemical Co. (St. Louis, MO). *p*-APMSF was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other inhibitors were obtained from Peptide Institute Inc. (Osaka, Japan).

2.3. *Radioimmunoassay (RIA)*

RIAs for ET and CTF were performed as described [6,7]. In RIA for ET, the cross-reactivity with big ET-1 was lower than 0.1%, whereas no cross-reactivity was observed with ET-1 in RIA for CTF. The antiserum used in the latter had a 100% cross-reactivity with big ET-1.

2.4. *Reverse-phase high performance liquid chromatography (RP-HPLC)*

RP-HPLC was performed using a Capcell-Pak $5C_{18}$-SG300 column (4.6 × 250 mm, Shiseido, Tokyo, Japan) eluted with a linear gradient from 0% to 35% CH_3CN in 0.02% trifluoroacetic acid (TFA) for 15 min, followed by isocratic elution at 35% CH_3CN in 0.02% TFA for 15 min and a linear gradient from 35% to 63% CH_3CN in 0.02% TFA for 15 min. The flow rate was 0.5 ml/min. Each fraction was evaporated and assayed for immunoreactive (IR)-ET and IR-CTF, using RIA.

2.5. *Peptides*

Porcine $ET\text{-}1_{1-21}$ and big $ET\text{-}1_{1-39}$ were obtained from Peptide Institute Inc. (Osaka, Japan). The CTF_{22-39} was prepared by solid phase synthesis. The homogeneity was confirmed by RP-HPLC and amino acid analysis.

3. RESULTS AND DISCUSSION

We first examined the effects of enzyme inhibitors on increases in IR-ET content observed during incubation of big ET-1 with cytosolic or membrane fraction at pH 7.0 for 6 h (Fig. 1). When big ET-1 was incubated with the cytosolic fraction, the IR-ET content was markedly increased (0.23 ± 0.02 to 4.81 ± 0.35 ng IR-ET/ml), and no appreciable alteration was observed, in the presence or absence of enzyme inhibitors. Incubation with the membrane fraction also resulted in a marked increase in the IR-ET content (0.23 ± 0.02 to 3.63 ± 0.32 ng IR-ET/ml), but the increased responses with this fraction were suppressed by the addition of enzyme inhibitors. When inhibitors were removed from the reaction system, one by one, it became evident that the removal of phosphoramidon, a metalloproteinase inhibitor [9], greatly increased the IR-ET content in the reaction mixture. This suggested that a phosphoramidon-sensitive metalloproteinase participates in the increase in IR-ET. No ET-immunoreactivity was detected in cytosolic or membrane fraction. When ET-1, instead of big ET-1, was incubated under the same conditions with each fraction, the IR-ET was slightly decreased, but there were no noticeable differences in the presence or absence of enzyme inhibitors, thereby suggesting that ET-1 was

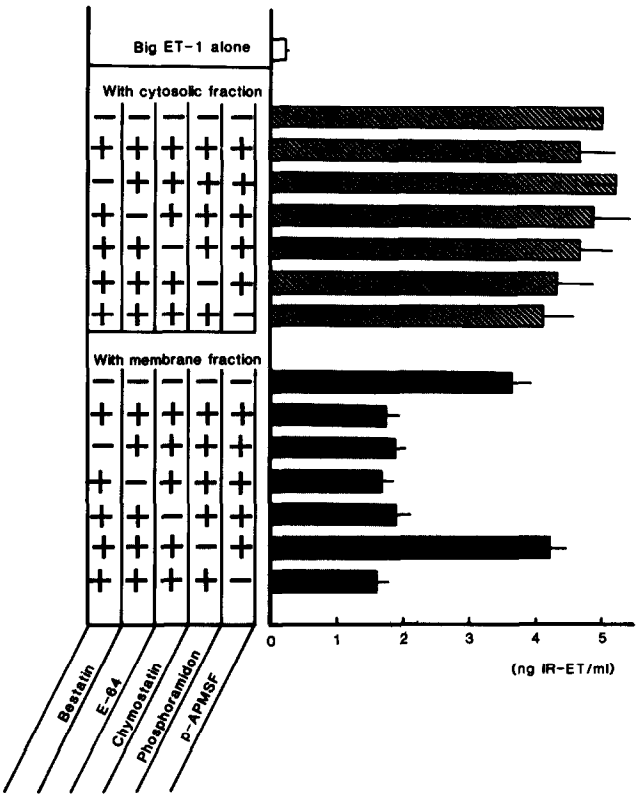

Fig. 1. Changes in IR-ET in the reaction mixture of big ET-1 with either cytosolic or membrane fraction. Incubation was carried out at pH 7.0 for 6 h at 37°C, in the absence or presence of enzyme inhibitors (bestatin, 10^{-5} M; E-64, 10^{-4} M; chymostatin, 2×10^{-6} M; phosphoramidon, 10^{-5} M; *p*-APMSF, 10^{-4} M). Each column and bar represents the mean ± SE from 6 separate experiments.

relatively stable during incubation at pH 7.0 for 6 h (data not shown).

Fig. 2 shows the pH sensitivity (A) and the time-course (B) of the increase in IR-ET during incubation of big ET-1 with cytosolic or membrane fraction. The optimum pH of the former and the latter cases was 7.0 and 6.5, respectively. In both cases, IR-ET increased linearly during 6 h after the start of incubation, and thereafter, the IR-ET content gradually decreased.

Using RP-HPLC coupled with RIAs for the CTF and ET, we characterized the IR-CTF and IR-ET in the reaction mixture of big ET-1 with each fraction. The elution profiles in case of the cytosolic fraction revealed one major IR-ET component corresponding to the elution position of synthetic ET-1 (Fig. 3A), whereas IR-CTF consisted of one major and one minor component corresponding to elution positions of synthetic porcine big ET-1 and CTF, respectively (Fig. 3B). Qualitatively similar results were obtained in case of the reaction mixture with the membrane fraction (Fig. 4A and B). The addition of phosphoramidon to the reaction mixture resulted in decreases in ET-1- and CTF-like materials accompanied by an increase in big ET-1-like materials (Fig. 4C and D). In all cases, the amounts of ET-1-like

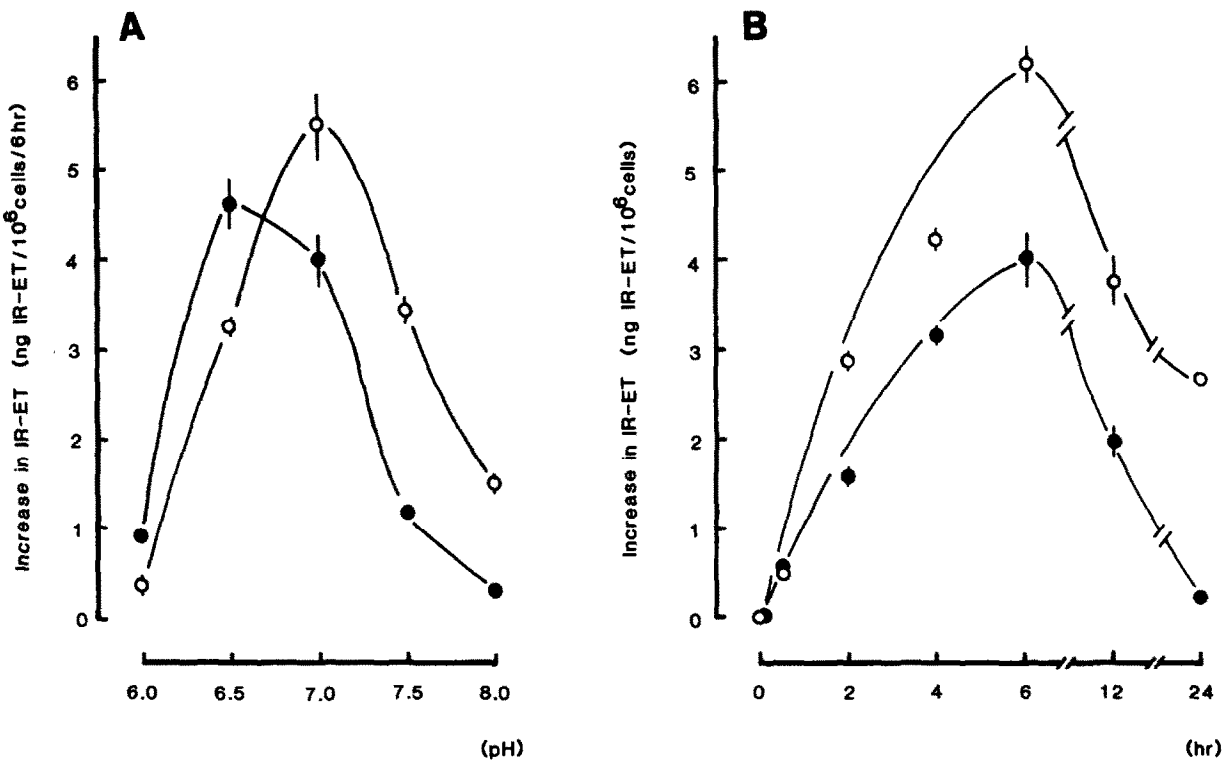

Fig. 2. Effects of pH on the increase in IR-ET during incubation of big ET-1 with either cytosolic or membrane fraction (A) and the time-course of the increased response (B). Incubation with the cytosolic fraction (○) was carried out in the absence of enzyme inhibitors. Incubation with the membrane fraction (●) was carried out in the presence of enzyme inhibitors, except for phosphoramidon. In experiments examining the pH sensitivity, the incubation was carried out for 6 h. The time-course was examined at pH 7.0. Each point and bar represents the mean ± SE from 4 separate experiments.

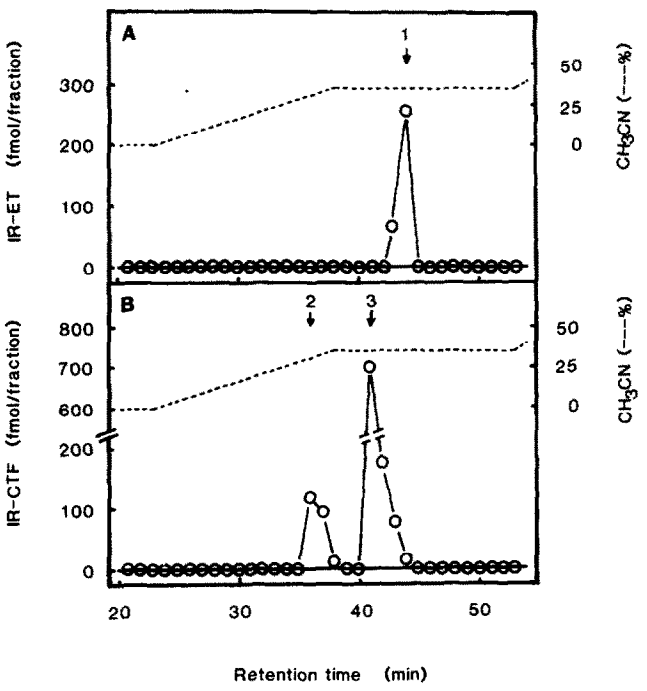

Fig. 3. RP-HPLC profiles of IR-ET (A) and IR-CTF (B) in the reaction mixture of big ET-1 with the cytosolic fraction. Incubation was carried out at pH 7.0 for 6 h in the absence of enzyme inhibitors. Arrows indicate the elution positions of ET-1 (1), CTF (2) and big ET-1 (3).

materials almost equalled those of CTF-like materials, on a molar basis. These results strongly suggest that the IR-ET observed by incubation of big ET-1 with either the cytosolic or membrane fraction is due to ET-1 converted from big ET-1 via a single cleavage between Trp^{21} and Val^{22}. In addition, we confirmed that phosphoramidon suppressed the membrane fraction-induced apparent conversion of big ET-1 to ET-1.

We examined effects of agents with the potential to inhibit the activity of metalloproteinase, on increases in IR-ET during incubation of big ET-1 with each fraction. Phosphoramidon (10^{-7}–10^{-4} M) suppressed dose-dependently the apparent converting activity of the membrane fraction. Metal chelators, such as EDTA and 1,10-phenanthroline (2×10^{-4} and 10^{-3} M), almost completely abolished the membrane fraction-induced apparent conversion (Fig. 5B). Thiorphan (10^{-7}–10^{-5} M), a specific inhibitor of neutral endopeptidase 24.11 [10], an enzyme classified as a metalloproteinase and widely distributed in the body in a membrane-bound and/or soluble form [11], had no suppressive action on the converting activity (Fig. 5B). Endopeptidase 24.11 has been reported to cleave peptides including enkephalins, kinins, atrial natriuretic peptide and substance P [11]. Since the concentrations of thiorphan we used are sufficient to produce a complete inhibition of endopeptidase 24.11 activity [10], the

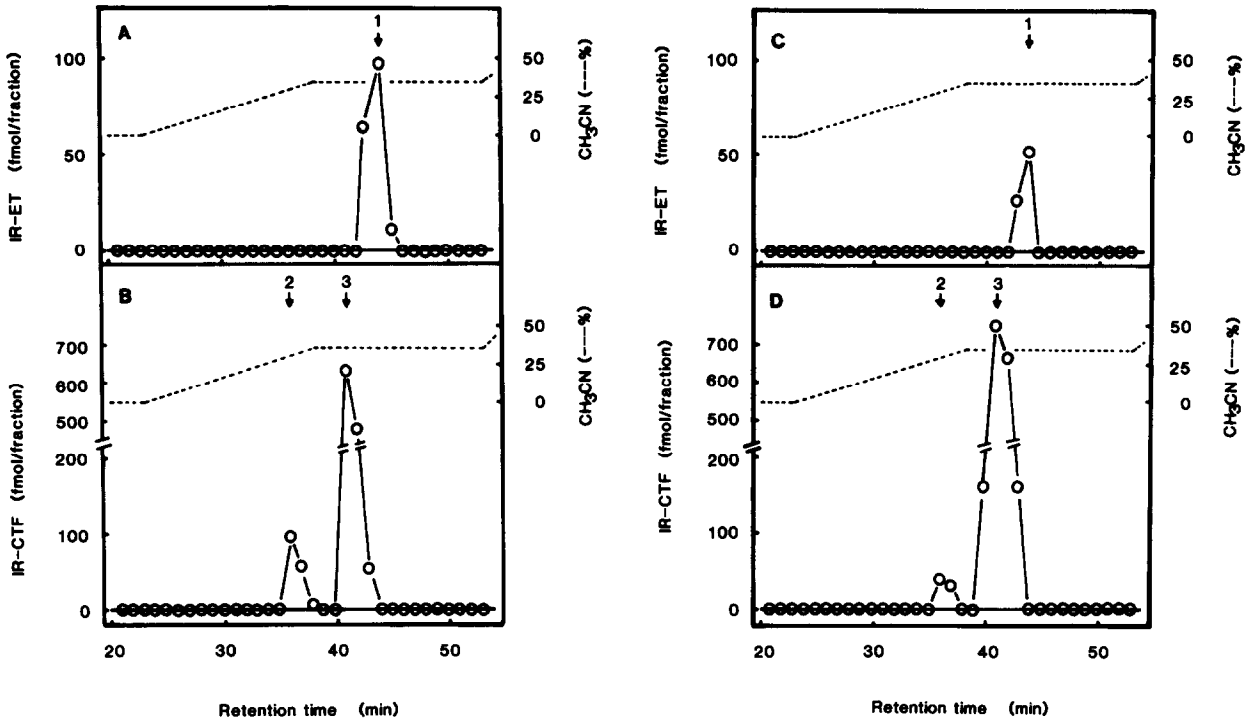

Fig. 4. RP-HPLC profiles of IR-ET (A,C) and IR-CTF (B,D) in the reaction mixture of big ET-1 with the membrane fraction. Incubation was carried out at pH 7.0 for 6 h in the absence (A,B) or presence (C,D) of 10^{-5} M phosphoramidon. Arrows indicate the elution positions of ET-1 (1), CTF (2) and big ET-1 (3).

phosphoramidon-sensitive enzyme in the membrane fraction is presumably not endopeptidase 24.11.

In contrast to results seen with the membrane fraction, phosphoramidon had no effect on the cytosolic fraction-induced apparent conversion. Thiorphan was also without effect. However, two metal chelators potently inhibited the converting activity of this fraction (Fig. 5A). Metal chelators inhibit both metallopro-

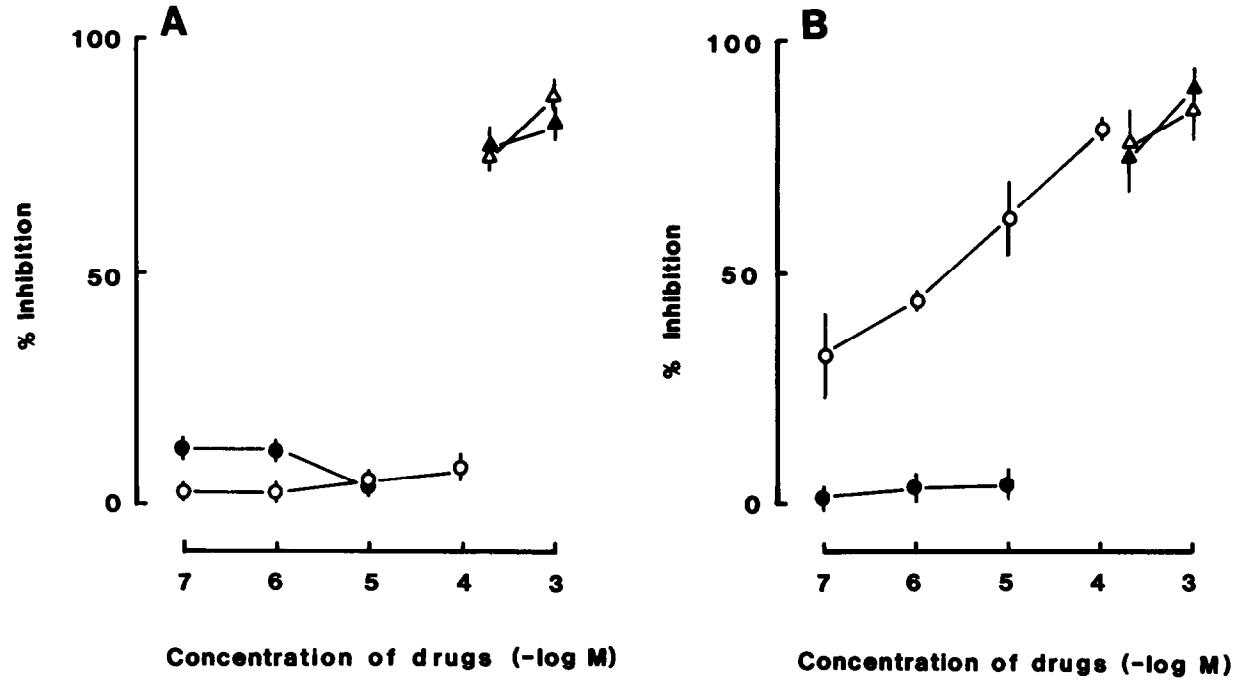

Fig. 5. Inhibitory effects of phosphoramidon (○), thiorphan (•), EDTA (▲) and 1,10-phenanthroline (△) on the increase in IR-ET during incubation of big ET-1 with either cytosolic (A) or membrane (B) fractions. Incubation was carried out at pH 7.0 for 6 h. Each point and bar represent the mean ± SE from 4 separate experiments.

teinases and metal-activated enzymes. The former enzymes contain metal ions (usually zinc) at the active center, whereas the latter enzymes usually contain cysteine or serine as the catalytic residues [12]. Accordingly, the metalloproteinases can often be distinguished from the metal-activated enzymes by the inhibition profile of enzyme inhibitors [12]. Taken together with our results (Fig. 1), the conversion seen with the cytosolic fraction also seems to be mediated by enzymes belonging to metalloproteinases.

The present study indicates that vascular ECs contain two types of metalloproteinases which convert big ET-1 to ET-1 via a single cleavage between Trp^{21} and Val^{22}. One is phosphoramidon-sensitive and exists in a membrane-bound form, whereas the other is phosphoramidon-insensitive and may be present in a soluble form. Most recently, we found that ET-1 can be generated by incubating big ET-1 with the EC extract at an acid pH. Since the generation was specifically inhibited by pepstatin-A, we considered that an aspartic proteinase may participate in the intracellular processing of big ET-1 in vascular ECs [7]. Thus, the conversion of big ET-1 in ECs is indeed complicated and may be mediated by more than one enzyme. We also obtained data that the i.v. administration of phosphoramidon to anesthetized rats markedly suppressed the big ET-1-induced hypertensive effect without affecting the hypertension induced by ET-1 [13]. In addition, the secretion of ET-1 from cultured ECs was abolished by the addition of phosphoramidon (Ikegawa et al., submitted). From these findings, it seems reasonable to consider that phosphoramidon-sensitive metalloproteinase which probably exists in a membrane-bound form is the most plausible candidate for big ET-1 converting enzyme, in vivo.

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